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Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

Normes OEPP
EPPO Standards

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

PM 7/40

oepp
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Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Diagnostic Protocols for Regulated Pests are intended to be used by National Plant Protection Organizations, in their capacity as bodies responsible for the application of phytosanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use, etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all diagnostic protocols:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable
- laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

References

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Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.
Quarantine pest: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Diagnostic Protocols for Regulated Pests provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Nineteen EPPO standards on diagnostic protocols have already been approved and published. Each standard is numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69

PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77

PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253

PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259

PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265

PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275

PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279

PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292

PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298

PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310

PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256

PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264

PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270

PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280

PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288

PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

Several of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'intercomparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostic protocols for regulated pests[1]
Protocoles de diagnostic pour les organismes réglementés

Globodera rostochiensis and *Globodera pallida*

Specific scope

This standard describes a diagnostic protocol for *Globodera rostochiensis* and *Globodera pallida*.

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2003-09.

Introduction

Globodera rostochiensis and *Globodera pallida* are the two species of potato cyst nematodes which cause major losses in potato crops (van Riel & Mulder, 1998). The infective juvenile nematodes only move a maximum of about 1 m in the soil. Most movement to new localities is by passive transport. The main routes of spread are infested seed potatoes and movement of soil (e.g. on farm machinery) from infested land to other areas. Infection occurs when the second-stage juvenile hatches from the egg and enters the root near the growing tip by puncturing the epidermal cell walls, and then internal cell walls, with its stylet. Eventually it begins feeding on cells in the pericycle, cortex or endodermis. The nematode induces an enlargement of root cells and breakdown of their walls to form a large, syncytial transfer cell. This syncytium provides nutrients for the nematode. Infested potato plants have a reduced root system and, because of the decreased water uptake, plant death can eventually occur.

Identity

Name: *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959
Synonyms: *Heterodera schachtii rostochiensis* Wollenweber, 1923; *Globodera schachtii solani* Zimmerman, 1927
Taxonomic position: Nematoda: Tylenchida: *Heteroderidae*
Bayer code: HETDRO
Phytosanitary categorization: EPPO A1 list No. 125, EU Annex designation I/A2
Name: *Globodera pallida* Stone, 1973
Synonyms: *Heterodera pallida* Stone, 1973
Taxonomic position: Nematoda: Tylenchida: *Heteroderidae*
Bayer code: HETDPA
Phytosanitary categorization: EPPO A1 list No. 124, EU Annex designation I/A2

Detection

Symptoms

Symptoms due to potato cyst nematodes are not specific. General symptoms include patches of poor growth in the crop, with plants sometimes showing yellowing, wilting or death of foliage; tuber size is reduced as a result, sometimes even when only minor symptoms are visible. However, many other causes can lead to these symptoms. Plants should therefore be lifted for a visual check on the presence of cysts and young females on the roots, or a soil sample should be taken for testing. Young females and cysts are just visible to the naked eye as tiny white, yellow or brown globes on the root surface (Web Figs 1 and 2). Detection by lifting plants is only possible during a very narrow time lapse and it is time-consuming. Soil testing is therefore the best way of determining the presence of potato cyst nematodes.

Statutory sampling procedures
Details of sampling and extraction methods can be found in EPPO Standard PM 3/30 (OEPP/EPPO, 1991).

Identification

The female colour at the appropriate stage can be used as an indication: a female which changes from white to yellow, then into a brown cyst, is *G. rostochiensis*, while one which changes

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Table 1 Range and mean values of measurements of *Globodera rostochiensis*, *G. pallida*, *G. tabacum* s.l., *G. achilleae* and *G. artemisiae*, range and mean values (Balwin & Mundo-Ocampo, 1991; Brzeski, 1998)

Species	J2 stylet Shape of anterior length (μm) surface of knob	Number of cuticular ridges	cyst between anus and vulval basin	Granek's ratio
G. rostochiensis	rounded	19–23 (21.8)	16–31 (> 14)	1.3–9.5 (> 3)
G. pallida	pointed	22–24 (23.8)	8–20 (< 14)	1.2–3.5 (< 3)
G. tabacum	pointed to concave	23–24	10–14	1–4.2 (< 2.8)
G. achilleae	rounded	24–26 (25)	4–11 (< 10)	1.3–1.9 (1.6)
G. artemisiae	rounded	18–29 (23)		0.8–1.7 (1.0)

from white directly to brown is *G. pallida*. Identification of cysts and other stages is in general based on a combination of morphological & morphometric characters and biochemical techniques. For light microscope identification, it is recommended to examine specimens mounted in fixative on microscope slides (Appendix 1).

Extraction procedures

The processes of extracting cysts from the soil can be various. Simple methods based on flotation can be as good as elutriation. For each method, a description is given in Appendix 2.

Morphology

Sedentary females: smoothly rounded with small projecting neck, no terminal cone present, diameter ± 450 μm, ranging in colour from white to yellow. Cysts are similar in shape, but have a tanned brown skin. Cuticle surface with zig-zag pattern of ridges, a distinct D-layer is present. The perineal area consists of a single circumfenetration around the vulval slit, perineal tubercles on crescents near vulva. Anus subterminal without fenestra, vulva in a vulval basin, underbridge and bullae rarely present (Web Fig. 3). Eggs retained in cyst, no egg-mass present. The non-sedentary second-stage juveniles are vermiform, annulated and tapering at both ends. Body length ranging from 445 to 510 μm, stylet length 19–25 μm, tail length 37–55 μm and a hyaline tail part of 21–31 μm. Use of a combination of cyst and second-stage juvenile characteristics is recommended for reliable identification. These stages are normally present in most soil samples infested with potato cyst nematodes.

G. rostochiensis and *G. pallida* are morphologically and morphometrically closely related (Stone, 1973a, b). The most important cyst differences can be obtained from the observation of the perineal area, i.e. number of cuticular ridges between vulva-anus and Granek's ratio (the distance from the anus to the nearest edge of the vulval basin, divided by vulval basin diameter). The most reliable second-stage juvenile characters are stylet length and stylet knob shape (Table 1). Overlap of values takes place, so care is needed. Web Fig. 4 presents some drawings of different stages of *G. rostochiensis* and *G. pallida*.

Biochemical techniques

As *G. rostochiensis* and *G. pallida* are morphologically closely related, many different biochemical techniques have been developed to separate the two species. Schots *et al*. (1992) were able to differentiate and quantify them using a set of three monoclonal antibodies. There were, however, cross reactivity problems between the antibodies for the two species.

Another powerful DNA-based approach to diagnostics involves the polymerase chain reaction (PCR). Using this technique, primers are developed that bind to two target sites on each DNA strand and replicate a specific region of DNA. Several scientists have developed PCR tests to separate the two potato cyst nematode species (e.g. Fleming *et al*., 1993; Mulholland *et al*., 1996; Shields *et al*., 1996; Zouhar *et al*., 2000). Isoelectric focusing (IEF) has proved to be sensitive enough to identify samples of potato cyst nematodes. During IEF, proteins are separated in a pH gradient and focused at the position in the gradient (the isoelectric point) where they become electrically neutral. Four species-specific proteins were located, pI 5.9 & 8.7 and pI 5.7 & 6.9. These proteins can be used to identify *G. rostochiensis* and *G. pallida* (Fleming & Marks, 1982; Karssen *et al*., 1995). Other techniques, such as RFLP analysis (Burrows & Boffey, 1986), diagnostic probes (Marshall & Crawford, 1987; Burrows & Perry, 1988), RAPD-based specific primers (Fullaondo *et al*., 1999) and dot-blotting with specific probes (Marshall, 1993) have also proved to be useful to distinguish the two species.

For a complete treatise on the use of immunology, protein electrophoresis, IEF and DNA, see Fleming & Powers (1998). A comparison of IEF, ELISA and PCR used for identification of potato cyst nematodes in field samples is made by Ibrahim *et al*. (2001).

Most techniques have, however, been developed especially to distinguish *G. rostochiensis* from *G. pallida* but have not (yet) been tested against species such as *G. achilleae*, *G. tabacum* or *G. mexicana*. This limitation should be noted. There may also be differences between European and non-European populations of the two species. Thiéry & Mugniéry (1996), Grenier *et al*., 2001) and Subbotin *et al*. (2000) made tried to use DNA-based techniques.

Routine identification of *G. rostochiensis* and *G. pallida* should preferably combine morphological and molecular methods (see Appendix 3 for molecular detection).

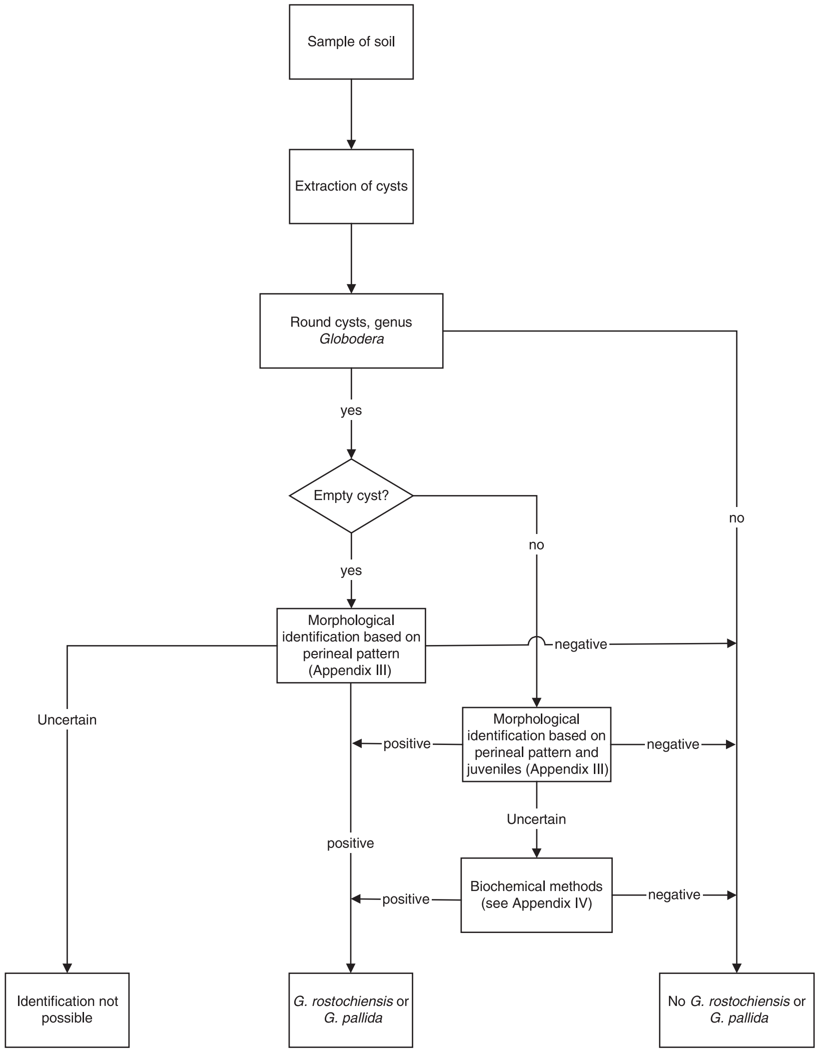

Fig. 6 Decision scheme for the detection and identification of *Globodera rostochiensis* and *G. pallida*.

Pathotypes

The term 'pathotype' is used by the International PCN Pathotype Scheme proposed by Kort *et al*. (1977), but is now considered too general. Populations cannot conclusively be identified in relation to these groups. In practice, the pathogenicity of populations can be tested on a set of cultivars used in each country, as a pragmatic way of dealing with the requirements of the EU Control Directive (EU, 1969), or equivalent requirements in other countries. This Directive is under revision and new requirements may be made, such as that each new introduction should be identified at 'pathotype' level.

Possible confusion with other species

The three other *Globodera* species which could cause confusion during identification of potato cyst nematodes in Europe are *G. achilleae* (Golden & Klindic, 1973) Behrens, 1975[2], *G. artemisiae* (Eroshenko & Kazachenko, 1972) Behrens, 1975 and *G. tabacum*. These first two species are not parasitic on potato, but recorded on the weeds *Achillea millefolium* and *Artemisia vulgaris*, respectively, in comparable agricultural areas. In North and Central America, the *G. tabacum* species complex [*G. tabacum tabacum* (Lownsbery & Lownsbery, 1954) Behrens, 1975; *G. tabacum solanacearum* (Miller & Gray, 1972) Behrens, 1975 and *G. tabacum virginiae* (Miller & Gray, 1972) Behrens, 1975] is found, and parasitizes tobacco, potato and solanaceous weeds. In southern Europe, *G. tabacum* is also present. See Appendix 3 and Web Fig. 5 for a morphometric and morphological comparison between potato cyst nematodes, *G. achilleae*, *G. artemisiae* and *G. tabacum*. See also Baldwin & Mundo-Ocampo (1991), Brzeski (1998) and Wouts & Baldwin, 1998) for more detailed information on other members of the *Heteroderinae* and

[2]Krall (1978) considered *G. millefolii* (Kirjanova & Krall, 1965) Behrens, 1975 as *species inquirenda*, as the description was based on a single female. Brzeski (1998) reported on *G. achilleae*: 'it may be conspecific with *G. millefolii*'. Additional research is needed to prove if *G. achilleae* is a junior synonym of *G. millefolii*.

identification keys. Under low magnification *Meloidogyne* and particularly *Heterodera* juveniles may appear similar to *Globodera* species.

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol and the decision scheme (Fig. 6) should have been followed.

Potato cyst nematodes do not induce galls on plant roots. For a positive diagnosis, second-stage juveniles and cysts should be obtained from the soil, plant roots or tubers. The cysts and second-stage juveniles should have the characters as described above for *G. rostochiensis* and *G. pallida*.

Report on the diagnosis

A report on the execution of the protocol should include:
- results obtained by the recommended procedures
- information and documentation on the origin of the infested material
- a description of the symptoms (if relevant)
- the number of individuals examined
- drawings or photographs of the following morphological features: cyst perineal area region, head (including stylet) and tail of second-stage juvenile
- measurements of the morphological features given in the description above
- applied biochemical techniques (Appendix 3)
- immunology, protein electrophoresis or DNA results (including primers used)
- an indication of the magnitude of the infestation
- comments as appropriate on the certainty or uncertainty of the identification

It is recommended to preserve specimens.

Further information

Further information on this organism can be obtained from: Dr L. den Nijs & Prof Dr G. Karssen, Plant Protection Service, PO Box 9102, 6700 HC Wageningen, the Netherlands. E-mail: L.J.M.F.den.Nijs@minlnv.nl

Acknowledgements

This protocol was originally drafted by: Dr L. den Nijs & Prof Dr G. Karssen, Plant Protection Service, PO Box 9102, 6700 HC Wageningen, the Netherlands.

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Appendix 1. Preparation of nematode specimens for microscope examination

For identification to species level of cysts (and second-stage juveniles) recovered from soil, specimens should be examined mounted in fixative on microscope slides. A good fixative for this purpose is TAF, which is a solution of 7 mL formalin (40% formaldehyde) and 2 mL triethanolamine in 91 mL distilled water. Second-stage juveniles are first killed by heating them for a few seconds in a small drop of water on a slide, until they just stop moving (longer heating will damage the specimens). An amount of double strength TAF or other fixative equal to the drop of water is immediately added or the juveniles are removed from the water and put into a new drop of warmed single-strength TAF. The preserved specimens are mounted on a glass slide with a fixed cover-slip and are examined at high-power light microscopy. The glass slide should be labelled with relevant data. To see some of the necessary diagnostic features, it may be necessary to use the highest power of the microscope (e.g. X100 with oil immersion) or to apply interference contrast microscopy. See Hooper (1985) and Golden (1986) for the preparation and morphological description, respectively, of vulval cones.

Appendix 2. Procedures for extracting cysts of *Globodera spp.* from soil

Extraction by flotation

These methods are based on the characteristic that dried cysts float. The dried soil sample is added to a beaker, flask or white dish that is filled with water. The suspension is well stirred. After 30 s to some minutes, depending on the soil type, the water is cleared and the suspension will only contain the floating organic debris and cysts. When a drop of detergent is added, the cysts will move to the edge and can be picked out by hand using a brush. Other ways are to decant carefully, or to use a paper strip around the beaker and to raise the water level so cysts adhere to it.

Several methods are in use to isolate the cysts from the debris (Turner, 1998), in particular the Fenwick can that has been in use for many years. It is usually made of metal and consist of a can, tapering towards the top and with a sloped base. The can has a sloping collar just below the rim. Beneath the outlet of the collar, a sieve is placed. The soil sample is put into a funnel on top of the can, water washes it through, the can fills with water, and cysts and organic matter will float on the water over the rim via the collar into the sieve.

The Schuiling centrifuge is a semiautomatic flotation method. The air-dried soil sample is placed in a transparent cylindrical container partly filled with water. The contents are swirled with a rotating two-pronged fork at 450–500 rev min^{-1}, creating a vortex causing cysts and other floating particles to be forced to the centre through a wire-mesh cylinder (1.5 mm). Cysts are collected on a sieve for further processing.

Extraction by elutriation

These methods are based on the difference in density of cysts by comparison with soil particles and can be used for wet soil. At the base of a (conical) column, water enters through a perforated tube at a constant rate (minimal 0.6 L min^{-1}). Soil is added into the column using a funnel. A small plate baffles the outlet of the funnel so that soil does not fall down the column too quickly. Via the overflow spout, the cysts are collected on a pair of sieves (53-µm aperture). The cysts are isolated from the debris as in the previous section.

Appendix 3. Recommendations for molecular detection

The following PCR methods are recommended for the identification of *G. rostochiensis* and *G. pallida* by use of species-specific primers: (1) Fullaondo *et al*. (1999), a PCR method based on species-specific primers designed from RAPD fragments; (2) Mulholland *et al*. (1996), a multiplex PCR-based method based on species-specific primers, which targeted ribosomal ITS1 and 5.8S genes; (3) Fleming *et al*. (1993), a relatively simple ITS-RFLP PCR method based on the well-known Vrain primers. These methods are useful for the identification of second-stage juveniles, white females, eggs and males. The DNA isolation methods are described in detail in the cited publications. See also Fleming & Powers (1998) for more information on different DNA extraction methods. Alternatively the High Pure PCR Template Preparation Kit (Roche, Almere, NL) can be used for DNA isolation, as this is a relatively easy method.

Primers

Fullaondo *et al*. (1999) decribed the following two species-specific primer sets for each *Globodera* species: (5′-GCAA-GCCCAGCGTCAGCAAC-3′and 5′-GAACATCAACCTC-CTATCGG-3′) for *G*. *rostochiensis* (resulting in a 315 bp amplicon); 5′-TGTCCATTCCTCTCCACCAG-3′ and 5′-CCGCTTCCCCATTGCTTTCG-3′ for G. *pallida* (resulting in a 798 bp amplicon). The primers can also be used in a mixture and have the same sized amplification products.

Mulholland *et al*. (1996) used one universal primer (5′-GCAGAAGGCTAGCGATCTTC-3′), and also specific primers for G. pallida (5′-GGTGACTCGACGATTGCTGT-3′) and *G*. *rostochiensis* (5′-TGTTGTACGTGCCGTACCTT-3′) (resulting in an amplicon of 391 bp for *G*. *pallida* and 238 bp for *G*. *rostochiensis*). The primers can also be used in a mixture and result in the same amplicons.

Amplification and analysis

For the composition of the reaction mixtures and PCR conditions, we refer to the above mentioned methods, where they are described in detail. Logically a negative control (no DNA template) and a positive control (DNA from reference culture) should be included in every experiment. Reference material can be obtained from the Plant Protection Service, Wageningen, the Netherlands.

Original photographs of gels should be taken and preserved.

Fig. 1. Potato roots infected by *G. rostochiensis* (Courtesy: Plant Protection Service, NL)

Fig. 2. Broken cyst with eggs of *G. pallida* (Courtesy: Plant Protection Service, NL)

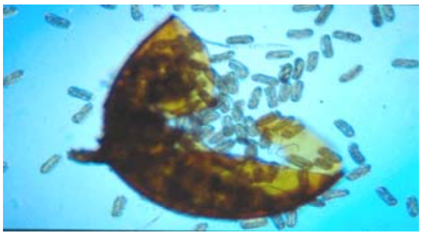


Fig. 3. The perineal region of a *Globodera* cyst (After Fleming and Powers, 1998).

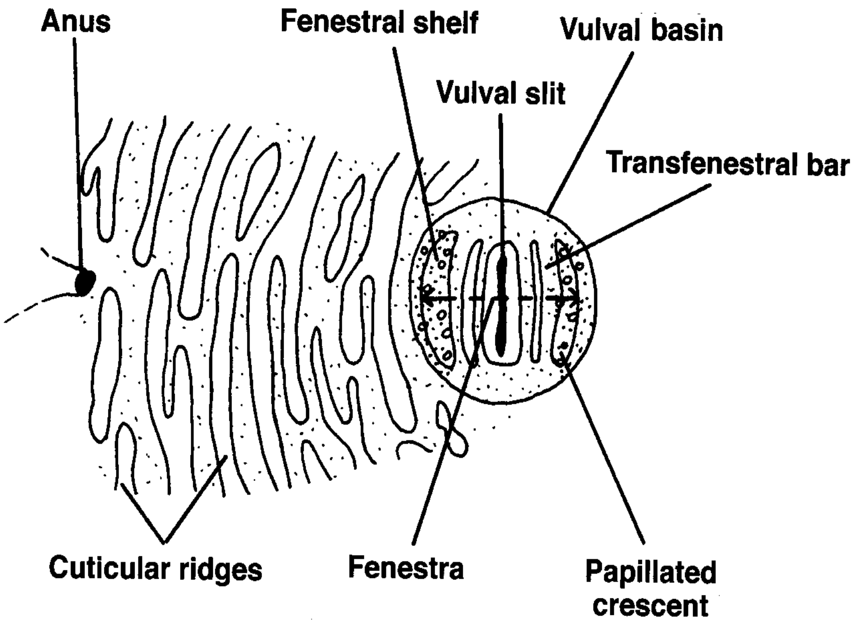
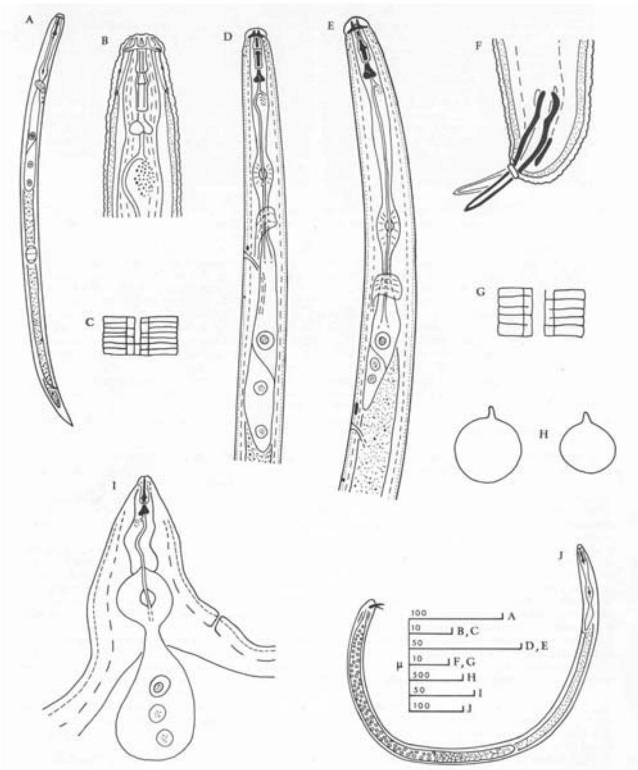


Fig. 4a: *G. rostochiensis*. **A.** Entire juvenile. **B.** Head region of 2nd-stage juvenile. **C.** 2nd-stage juvenile lateral field, mid-body. **D.** Pharyngeal region of 2nd-stage juvenile. **E.** Pharyngeal region of male. **F.** Tail of male. **G.** Lateral field of male, mid-body. **H.** Entire cysts. **I.** Head and neck of female. **J.** Entire male. (After: C.I.H. Descriptions of Plant-Parasitic Nematodes, Set 2, No. 16).

Fig. 4b: *G. pallida* 2[nd]-stage juvenile. **A.** Entire. **B.** Anterior. **C.** Head. **D.** Tail. **E.** Lateral field mid-body region. **F.** lateral field tail. **G.** Head *en face* at level of lips. **H.** Head *en face* at level of base. (After: Stone, 1972).

a



b

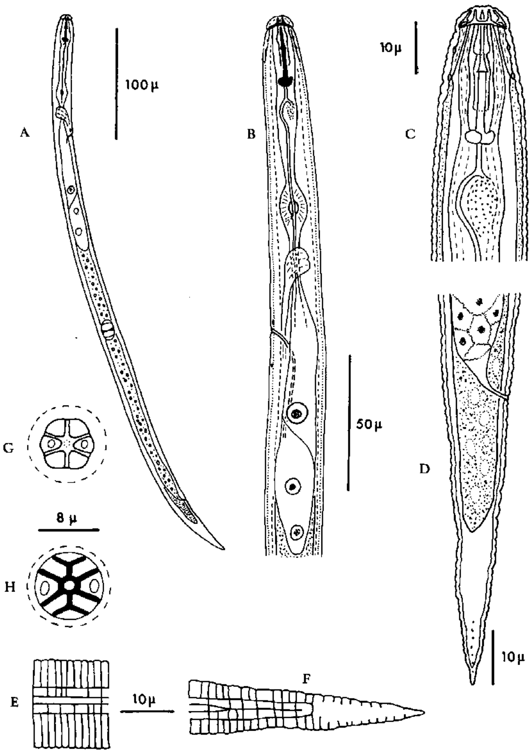


Fig. 5. A: Perineal measurements for *Globodera* identification. **B:** Vulval-anal ridge patterns for four *Globodera* species. **C:** Stylets from juveniles of four species of *Globodera* (After Fleming and Powers, 1998).